Scientia Horticulturae 217 (2017) 1–7

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Regulation of ascorbic acid metabolism in response to different temperatures in citrus juice sacs *in vitro*

Witchulada Yungyuen[a,b], Gang Ma[a], Lancui Zhang[a], Kazuki Yamawaki[a], Masaki Yahata[a], Satoshi Ohta[c], Terutaka Yoshioka[c], Masaya Kato[a,*]

[a] Department of Biological and Environmental Sciences, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan
[b] The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan
[c] Department of Citrus Research, NARO Institute of Fruit Tree Science, Okitsunakacho, Shimizu, Shizuoka 424-0292, Japan

ARTICLE INFO

Article history:
Received 21 September 2016
Received in revised form 6 December 2016
Accepted 12 January 2017
Available online 21 January 2017

Keywords:
AsA content
Gene expression
High temperature
Low temperature
Regulatory mechanism

ABSTRACT

To elucidate the regulation of ascorbic acid (AsA) metabolism in response to temperatures, three varieties of citrus juice sacs (Valencia orange, Lisbon lemon, and Satsuma mandarin) in *in vitro* culture system were subjected to different temperatures (10 °C, 20 °C, and 30 °C). The AsA accumulation was induced at 10 °C, whereas it was not significantly affected at 30 °C as compared with the control at 20 °C in juice sacs of the three citrus varieties. The enhancement of AsA accumulation at 10 °C was regulated by the expression of genes in AsA biosynthetic, oxidation, and regeneration pathway. In Valencia orange and Satsuma mandarin, the higher expression of *CitVTC4* gene in biosynthetic pathway, and the higher expression of *CitMDAR1* and *CitMDAR2* genes in regeneration pathway, together with the lower expression of *CitAO* gene in oxidation pathway contributed to the higher AsA level at 10 °C. In Lisbon lemon, the higher expressions of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes in biosynthetic pathway and the lower expression of *CitAO* gene in oxidation pathway contributed to the higher AsA level at 10 °C.

1. Introduction

Ascorbic acid (AsA) has been revealed to participate in several physiological processes in plants, such as co-factor of many key enzymes, and regulating plants defense mechanism (Horemans et al., 2000). In addition, AsA is also beneficial to human health. The consumption of AsA improves human immunity and reduces the risk of oxidative stress related illness, including some cancers and cardiovascular disease (Du et al., 2012). Plants are able to synthesize AsA. In contrast to most plants, humans cannot synthesize AsA. The major source of AsA for human diet is fruits and vegetables. Recently, some attempts have been made to increase nutritional value in fruits and vegetables by enhancing the AsA content (Bulley et al., 2009, 2012).

AsA biosynthesis consists of four distinct pathways. As shown in Fig. 1, L-galactose pathway is a main AsA biosynthetic route in higher plants (Wheeler et al., 1998). This pathway comprises six enzymatic steps to synthesize AsA including, GDP-D-mannose pyrophosphorylase (GMP, VTC1), GDP-D-mannose 3′,5′-epimerase (GME), GDP-L-galactose phosphorylase (GGP, VTC2), L-galactose-1-phosphate phosphatase (GPP, VTC4), L-galactose dehydrogenase (GalDH) and L-galactono-1,4-lactone dehydrogenase (GLDH), respectively. Then, AsA are further oxidized by ascorbate oxidase (AO) and ascorbate peroxidase (APX) into monodehydroascorbate (MDA). MDA can either be regenerated back into AsA by monodehydroascorbate reductase (MDAR) or non-enzymatic disproportionate to dehydroascorbate (DHA). After that, DHA can be recycled back into AsA by dehydroascorbate reductase (DHAR), using reduced glutathione (GSH) as a reducing substance. In ascorbate-glutathione cycle, the oxidized glutathione (GSSG) is recycled back into reduced glutathione (GSH) by glutathione reductase (GR) (Noctor and Foyer, 1998; Linster and Clarke, 2008).

Citrus fruits are rich in AsA content as compared to other commercial fruits and vegetables (Cruz-Rus et al., 2012). In citrus fruit, a major AsA biosynthetic route is L-galactose pathway (Yang

Abbreviations: AsA, ascorbic acid; VTC1, GDP-D-mannose pyrophosphorylase (GMP); GME, GDP-D-mannose 3′,5′-epimerase; VTC2, GDP-L-galactose phosphorylase (GGP); VTC4, L-galactose-1-phosphate phosphatase (GPP); GaLDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; AO, ascorbate oxidase; APX, ascorbate peroxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

* Corresponding author.
E-mail address: amkato@ipc.shizuoka.ac.jp (M. Kato).

http://dx.doi.org/10.1016/j.scienta.2017.01.021
0304-4238/

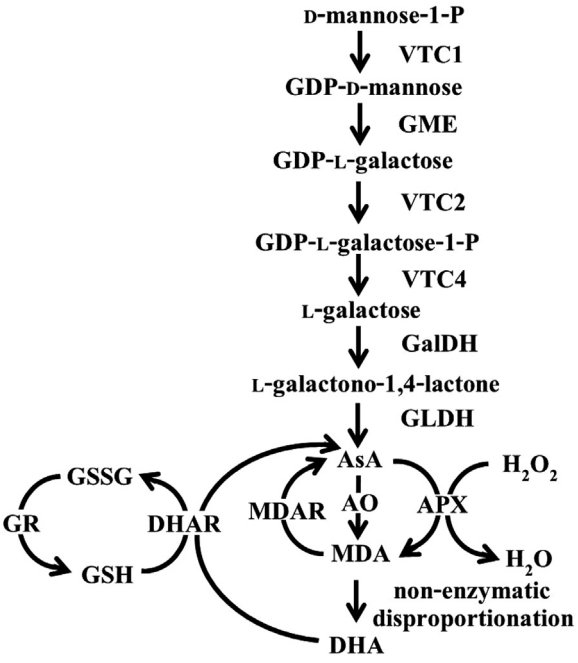

Fig. 1. The biosynthetic, oxidation, and regeneration pathway of AsA metabolism in plants. VTC1, GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose 3′,5′-epimerase; VTC2, GDP-L-galactose phosphorylase; VTC4, L-galactose-1-phosphate phosphatase; GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; AO, ascorbate oxidase; APX, ascorbate peroxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase.

et al., 2011; Alós et al., 2014). A wide variation of AsA content in the fruits was depended on various factors, such as varieties, tissues, and stages of fruit development (Lee and Kader, 2000; Li et al., 2008, 2009). Oranges and lemons had a higher AsA content, followed by grapefruits and mandarins among citrus genus (Escobedo-Avellaneda et al., 2014). Zhang et al. (2015) studied AsA accumulation in Valencia orange, Lisbon lemon, and Satsuma mandarin during the ripening progress. It was found that AsA accumulation during ripening process was different in the three citrus varieties. AsA content remained constant and significantly lower in Satsuma mandarin than that of the other two citrus varieties. The transcriptional balances in AsA biosynthesis, oxidation, and regeneration contributed to the different patterns of AsA accumulation during the ripening process. In addition, Alós et al. (2014) found that AsA accumulation was increased in the flavedo, whereas it was decreased in the pulp during fruit ripening. The results indicated that the accumulation of AsA in citrus fruits was independently regulated in each fruit tissues.

AsA content was also affected by several environmental factors. AsA accumulation was strongly affected by climatic conditions, including light and temperature (Massot et al., 2013). In citrus, light stimulated the accumulation of AsA, and the level of AsA was depended on the quality of light. The red LED light was not efficient to increased AsA amount, whereas the blue LED light notably boosted up AsA amount in juice sacs of citrus fruits. It was revealed that the up-regulation of genes in AsA biosynthetic and regeneration pathway by the blue LED light enlarged the AsA amount in juice sacs of citrus fruits (Zhang et al., 2015). Temperature significantly influenced on a number of physiological processes in plants. Low temperature has been reported to promote the plant growth rate in varieties of temperate fruits and vegetables. In addition, it also induced the accumulation of secondary metabolites in plants, including AsA (Proietti et al., 2009). A number of plants accumulated more AsA when they were grown under low field temperature than high field temperature, such as kiwifruit (Richardson et al., 2004), broccoli (Schonhof et al., 2007), spinach (Proietti et al., 2009) and tomato (Gautier et al., 2013). The previous researches reported that temperature strongly affected the biosynthetic, oxidation, and regeneration pathway in AsA metabolism. Massot et al. (2013) found that low temperature triggered the expression of AsA biosynthetic genes, whereas high temperature did not in tomato fruit during off-vine ripening. Rivero et al. (2004) also found that high temperature triggered the oxidation process, which was responsible for the loss of AsA content in tomato fruits when the temperature increased. Furthermore, in the last decade, some researches revealed the important of regeneration pathway in maintaining AsA level in plants (Chen et al., 2003; Gallie, 2013). The previous study found that the limitation of AsA regeneration by high temperature contributed to the reduction of AsA amount in tomato fruits (Massot et al., 2013).

To date, molecular basis underlying the accumulation of secondary metabolites in response to different environmental factors is becoming an attractive scientific research in citrus fruits. However, the regulation of AsA accumulation in response to different temperature conditions in citrus fruits remains unclear. In this study, the influences of temperature on AsA metabolism were elucidated in citrus. AsA quantification and the gene expression in AsA metabolism pathway were carried out in the three citrus varieties (Valencia orange, Lisbon lemon, and Satsuma mandarin) in *in vitro* cultured system at different temperatures (10 °C, 20 °C, and 30 °C). The results of this study contributed to increasingly clarify the molecular mechanism regulating AsA accumulation in response to different temperatures, which will provide new strategies to increase AsA content in citrus.

2. Materials and methods

2.1. Plant materials

In the present study, Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*C. sinensis* Osbeck), and Lisbon lemon (*C. limon* Burm.f.) were used as materials. The fruits approximately 4–5 cm in fruit diameter in the immature green stage were harvested randomly in the citrus trees. Satsuma mandarin was harvested from Fujieda Farm, Shizuoka, Japan. Valencia orange and Lisbon lemon were harvested from NARO Institute of Fruit Science, Department of Citrus Research, Okitsu, Shizuoka, Japan.

2.2. In vitro *culture system and temperature treatments*

Three varieties of citrus juice sacs were used in culture system with the published method of Zhang et al. (2012). The endocarp side up of juice sacs was cultured on 10 mL of Murashige and Skoog (MS) medium in culture tubes (22 mm × 120 mm). Sucrose (10% w/v) and agar (1% w/v) were added into medium and adjusted the pH to 5.7. Then, the medium was autoclaved. The juice sacs of citrus were cultured for two weeks under the same condition at 20 °C, and then cultured for two weeks under different temperature conditions at 10 °C, 20 °C, and 30 °C. The juice sacs were harvested at the fourth week of culture system by liquid nitrogen and stored at −80 °C.

2.3. AsA determination

AsA content in citrus juice sacs was measured by HPLC in three replications with the published method of Ma et al. (2014) and Zhang et al. (2015). 0.5 g of juice sacs sample and 4 mL of extraction buffer (3% metaphospholic acid and 8% acetic acid) were homogenized, and centrifuged at 14,000 × *g* for 20 mins. The supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA, USA), and 0.45 μM nylon filter (Advantec, Tokyo, Japan), respectively.